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ELECTROCHEMICAL IMMOBILISATION OF ENZYMES

PART II. GLUCOSE OXIDASE IMMOBILISED IN POLY-*N*-METHYLPYRROLE

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ABSTRACT

Electrochemical polymerisation of *N*-methylpyrrole in buffered aqueous solution containing the enzyme glucose oxidase produces adherent films at the electrode surface containing the active enzyme. Electrodes prepared in this manner can be used to detect glucose in solution in the range 0–0.22 mol dm^{-3}. The observed response from such electrodes as a function of glucose concentration, enzyme loading, and film thickness are in excellent agreement with theory. From a comparison of the experimental results and the theory we are able to characterise the enzyme kinetics and diffusion within the conducting polymer film. The results are consistent with a model in which the hydrogen peroxide produced by the enzymatic reaction reacts at the electrode surface and not at the polymer itself.

INTRODUCTION

In our previous paper [1] we presented a theoretical analysis of the transport and kinetics of substrate and products in an immobilised enzyme layer at an electrode. In our analysis we distinguished between electrochemical detection of the product at the electrode and throughout the film.

In this paper we present experimental results for the immobilisation of an enzyme by electrochemical polymerisation. This approach has a number of advantages. Firstly, the spatial distribution of the immobilised enzyme can be readily controlled. Secondly, by using electrically conducting polymers the thickness of the enzyme film may be varied and is easily controlled. Thirdly, it should be simple to build up multilayer structures using different enzymes and different polymers. Finally, it should be possible to modulate enzymic activity by changing the redox state of the polymer.

Foulds and Lowe [2] have recently published results for the immobilisation of glucose oxidase in polypyrrole films grown on printed platinum substrates. In this

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paper we report our results for the electrochemical immobilisation of glucose oxidase in films of poly-*N*-methylpyrrole. Our results are analysed using a model of the immobilised enzyme electrode response. On the basis of this analysis we are able to characterise the rate of the enzymatic reaction in the conducting polymer film. This type of detailed kinetic analysis is essential for the rational design and optimisation of immobilised enzyme electrodes.

Electrochemical oxidation of *N*-methylpyrrole is known to produce adherent, conducting films at the electrode surface when carried out in aqueous [3] or non-aqueous solution [4]. In order to use this approach for the immobilisation of an enzyme it is important to ensure that the polymerisation conditions do not denature the enzyme. Thus it is advantageous to carry out the reaction in aqueous solution. The majority of studies of conducting polymer formation in aqueous solution have used unbuffered media. As a consequence the pH at the electrode surface is not controlled during the polymerisation and is likely to become significantly more acidic than the bulk due to protons liberated in the course of the reaction. We have therefore chosen to carry out all our studies in buffered aqueous solutions to avoid possible adverse effects on the enzyme activity.

In the studies reported here glucose oxidase was chosen as a model system. This is a suitable choice since the homogeneous enzyme kinetics are well characterised [5,6], the enzyme is readily available in a pure form, and it is reasonably stable. The enzyme-catalysed reaction of glucose may be written as follows:

$$\text{glucose} + \text{GOD}(\text{FAD}^+) \rightarrow \text{gluconolactone} + \text{GOD}(\text{FADH}_2) \quad (1)$$

$$\text{O}_2 + \text{GOD}(\text{FADH}_2) \rightarrow \text{H}_2\text{O}_2 + \text{GOD}(\text{FAD}^+) \quad (2)$$

where $\text{GOD}(\text{FADH}_2)$ represents the reduced form and $\text{GOD}(\text{FAD}^+)$ the oxidised form of the enzyme. With the enzyme immobilised at the electrode surface in a conducting polymer film we can follow these reactions by the electrochemical detection of the hydrogen peroxide produced. This can occur, in principle, in two ways: either the hydrogen peroxide may be oxidised at the surface of the underlying electrode, or it may be oxidised on the conducting polymer itself. It is also possible that some hydrogen peroxide may be lost by disproportionation within the film: however, this is likely to be a very inefficient process and we do not consider this a significant loss mechanism in our experiments.

EXPERIMENTAL

Instrumentation

All electrochemical studies were carried out using a purpose-built potentiostat/galvanostat and voltage generator in conjunction with a Bryans 60000 xy/t recorder. Rapid growth transients were initially recorded on a Gould OS4020 digital storage oscilloscope with roll-out facility. Rotating disc electrodes and rotator were supplied by Oxford Electrodes Ltd. All measurements were carried out in an undivided jacketted cell using a platinum gauze counter electrode and a saturated

calomel reference. Solutions were thermostatted at 25.0 ± 0.1° C. Working electrodes were polished with 1.0 and 0.3 μm alumina slurry and washed copiously before each experiment. Enzyme activities were determined using standard assay procedures with a Rank Brothers oxygen electrode.

Enzyme immobilisation

Enzyme films were grown either potentiostatically or galvanostatically, see below, at a stationary platinum disc from a deoxygenated solution of glucose oxidase in 0.1 mol dm^{-3} tetraethylammonium tetrafluoroborate buffered at pH 7.2 with 0.15 mol dm^{-3} phosphate containing 0.05 mol dm^{-3} *N*-methylpyrrole. Electrodes were washed by rotating them in buffer solution for 4 min to remove any weakly bound enzyme.

Solutions and reagents

All solutions were prepared using water from a Millipore Milli-Q system with AnalaR grade reagents unless otherwise stated. Buffer solutions were prepared by titration to the desired pH and were stored below 4° C. Stock solutions of glucose in buffer were prepared from AnalaR D-glucose (Fisons) and the anomers were allowed to equilibrate for at least 24 h before use. *N*-methylpyrrole (Aldrich) was freshly distilled before use. Glucose oxidase (E.C. 1.1.3.4) was supplied by Boehringer (grade II) or was a gift from Genetics International.

Procedure

Glucose responses were measured at a rotating electrode (9 Hz) at 950 mV vs. SCE. The background current was first allowed to stabilise. This typically took 40 min to fall to < 100 nA the first time an enzyme electrode was used but took only about 5 min thereafter. The glucose concentration was altered by adding a stock solution of glucose to a known volume of buffer using a microsyringe and allowing the solution to mix.

RESULTS

Growth of polymer films

In order to develop a general method for enzyme immobilisation using electrochemical polymerisation it is important to carry out the procedure under conditions which will not denature the enzyme. For this reason we have chosen to use buffered aqueous solutions for the immobilisation step. This is probably not essential for glucose oxidase which is acid tolerant [7].

To the best of our knowledge no studies of the electropolymerisation of pyrrole or *N*-methylpyrrole in buffered aqueous solutions have been reported. In unbuffered solutions the protons released in the polymerisation step [8] will lead to a decrease in the local pH. Comparison of polymerisation in buffered solution at pH 7.2 and in unbuffered solution shows that careful potential control is much more important for the buffered case. Figure 1 shows a typical set of transients for the growth of

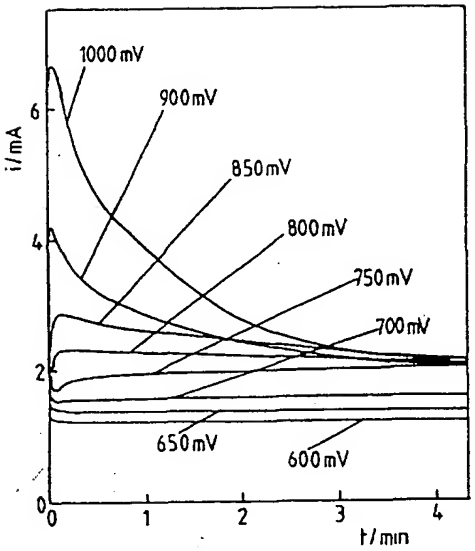

Fig. 1. Current transients for the potentiostatically controlled growth of poly-*N*-methylpyrrole films at a series of potentials.

polymer films at a clean electrode. Below 700 mV very little current is passed and the films are powdery and non-adherent. Above 950 mV initial currents are large and the resulting films are thick, uneven and tend to lift off of the electrode. Between these two potentials the films are even and adherent. In all cases these desirable characteristics are associated with transients of the type seen in Fig. 1 for this potential range.

In the presence of the enzyme the growth transients are of the same general form as those in Fig. 1 except that the currents are typically a factor of two larger. Again the best films, as defined by their physical appearance and adherence, are obtained by holding the electrode at potentials between 750 and 800 mV. In order to probe the mechanism of the film growth and the possible effect of the enzyme on this process we have studied the short-time transients. Typical results for a range of potentials are shown in Fig. 2. The results obtained with and without enzyme present are in excellent agreement with those of Pletcher and co-workers [3] who investigated the growth of *N*-methylpyrrole from unbuffered aqueous solutions. In accord with their findings, the best fits to the data were obtained for plots of i vs. t^2. The natural logarithm of the slopes of these plots vs. the potential were linear with a slopes of $(38\ \text{mV})^{-1}$ and $(77\ \text{mV})^{-1}$ for the cases with and without enzyme respectively. These results indicate that the growth mechanism is consistent with instantaneous nucleation of the polymer and three-dimensional growth.

The use of galvanostatic control in the immobilisation of the enzyme was also investigated. Films produced in this way were found to be essentially identical to those grown under potentiostatic control. The optimum conditions for the growth of poly-*N*-methylpyrrole films containing glucose oxidase are given in Table 1. In the

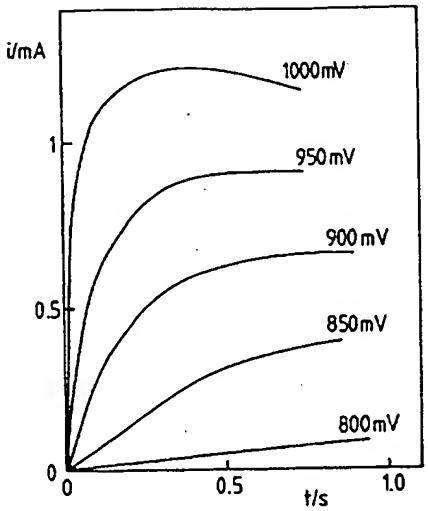

Fig. 2. Short-time current transients for the initial phase of polymer growth from a solution containing *N*-methylpyrrole and glucose oxidase at a variety of potentials.

work which follows all films were prepared using one of the two methods given in the table.

Responses to glucose

Figure 3 shows a typical set of responses to the addition of glucose. It is satisfactory that the response times for the electrode are of the order of 5 s. The inset in Fig. 3 shows a plot of the responses as a function of the glucose concentration. The response is linear in glucose up to concentrations well in excess of the Michaelis constant for the homogeneous enzyme. Indeed the electrode continues to respond to glucose additions above 0.22 mol dm^{-3} glucose.

Using our theoretical model [1], we can analyse the variation of the response of the electrode with the film thickness and the enzyme loading. The film thickness was varied by changing the charge passed during polymer growth. Figure 4 shows a plot of the slope of the calibration curve against the charge passed in growing the film;

TABLE 1

Optimum conditions for growth of polymer films containing glucose oxidase

Method [a]	Conditions	Time/min
Potentiostatic (P)	Step from open circuit to 800 mV Typical current density (400 mA/cm^2)	2–8
Galvanostatic (G)	Step from $i = 0$ to 300 mA/cm^2 Typical voltage 750 mV	2–8

[a] All solutions 0.05 mol dm^{-3} *N*-methylpyrrole in 0.15 mol dm^{-3} phosphate pH 7.2. 0.1 mol dm^{-3} NEt_4BF_4 containing 1 mg/ml glucose oxidase (160 U/ml) degassed with nitrogen.

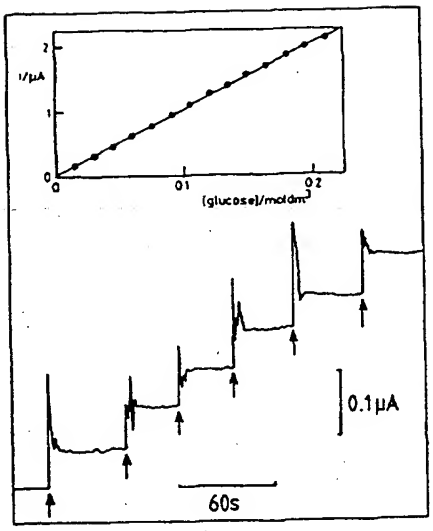

Fig. 3. The response of a glucose oxidase electrode to additions of glucose (marked by the arrows) to the bulk solution; the electrode was rotated at 9 Hz. $E = 0.95$ V. The inset shows a typical plot of the current as a function of the concentration of glucose in solution

under these conditions the electrode response was independent of the rotation speed. Clearly there is an optimum film thickness which maximises the sensitivity of the electrode to glucose. This type of behaviour is predicted by the theoretical analysis; eqn. (19) of our previous paper [1] gives

$$i/s_\infty = nFAD_S K\left[1 - \operatorname{sech}(1/X_K)\right]/l \tag{3}$$

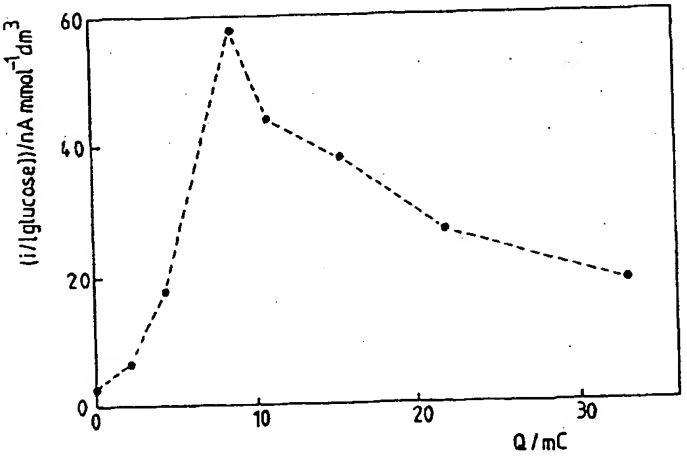

Fig. 4. Plot of the slope of the glucose calibration curve against the charge passed to grow the film containing the enzyme. All films were grown potentiostatically as described in Table 1.

where i is the current due to oxidation of hydrogen peroxide. s_∞ is the bulk concentration of glucose, D_S and K are respectively the diffusion coefficient and partition coefficient of glucose in the film, l is the film thickness, and X_K is the kinetic length given by

$$X_K = (D_S K_M / k_{cat} e_\Sigma)^{1/2} \tag{4}$$

In eqn. (4) k_{cat} is the first-order rate constant for the reaction of the enzyme substrate complex, e_Σ is the concentration of enzyme in the film, and K_M is the Michaelis constant. We can compare the experimental results to theory by constructing a log/log plot of the slopes of the experimental calibration curves against the charge passed in film growth and comparing this to a dimensionless log/log plot of eqn. (3). This is shown in Fig. 5. From the figure we can see that there is excellent agreement between the data for thick films and the theory. For the thinner films, however, the observed responses are always less than predicted. We shall return to this point below.

Equation (3) also predicts the effect of variation in enzyme concentration on the response. We have investigated the response as a function of the concentration of enzyme in the growth solution. When $1/X_K > 1$ we find that the electrode response is independent of the enzyme concentration as predicted by eqn. (3). However, when

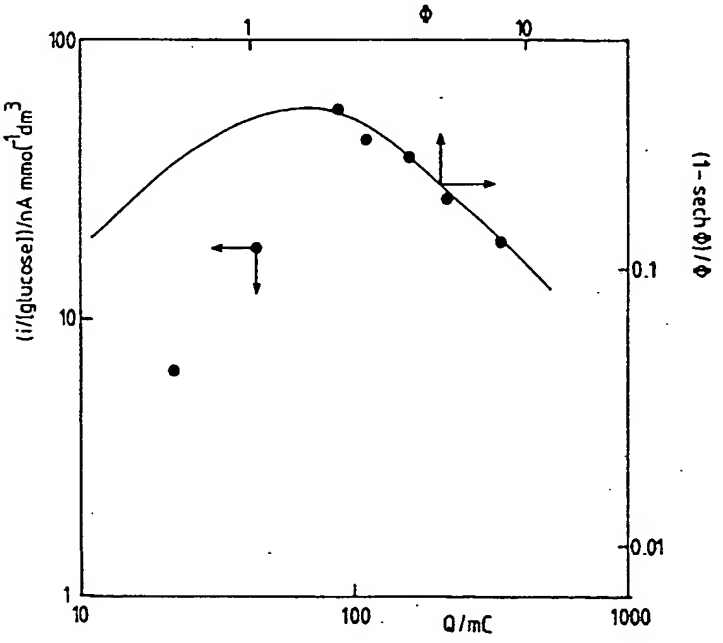

Fig. 5. Comparison of the experimental variation of the response of the electrode to added glucose as a function of film thickness with the theoretical expression. The points are the experimental results and the solid curve is calculated from eqn. (3). The values of the kinetic parameters derived from the fit are given in Table 2.

$1/X_K \ll 1$ we find that the response varies with enzyme loading, as predicted, and that the response becomes dependent on the rotation speed of the electrode at high enzyme concentrations. Figure 6 shows that as the rotation speed increases, the response decreases and approaches a plateau. This behaviour is consistent with the concentration polarisation of hydrogen peroxide. Thus at low rotation speeds the H_2O_2 builds up in the diffusion layer decreasing the loss of the material to the bulk; at high rotation speeds H_2O_2 is rapidly removed from the electrode surface. We can model this by adapting the boundary conditions in our previous treatment. Under these conditions we replace eqn. (10) in the previous paper by

$$\text{at } x = 1 \qquad \partial b/\partial x = -k'_D b_1 / K_B D_{B'} \tag{5}$$

where b_1 is the concentration of hydrogen peroxide in the film at the surface. K_B is the partition coefficient for H_2O_2 into the film. $D_{B'}$ is the diffusion coefficient for H_2O_2 in the solution, and k'_D is the mass transfer rate constant for removal of hydrogen peroxide, given by [9]

$$k'_D = 1.554 D_{B'}^{1/3} \nu^{1/6} W^{1/2} \tag{6}$$

Solution of eqn. (A8) of ref. 1 with this boundary condition gives the following expression for the flux of hydrogen peroxide detected at the electrode

$$f_{obs} = (j_S + j_B l k'_D / D_{B'}) / (1 + l k'_D / D_{B'}) \tag{7}$$

where j_S and j_B are given by eqns. (16) and (19) of ref. 1 respectively, and f_{obs} is the observed current. This expression reduces to our previous result for j_B when k'_D is large and gives the corresponding result for j_S when k'_D is very small. When

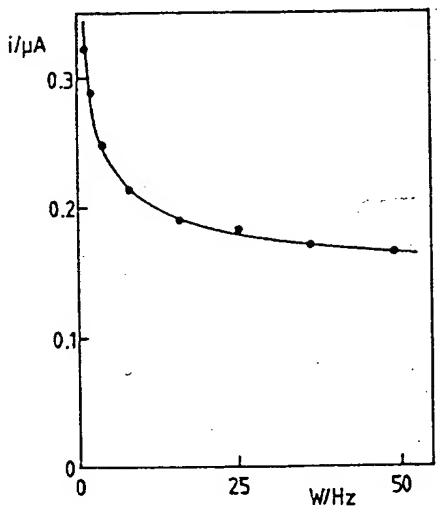

Fig. 6. The variation of the electrode response with the rotation speed for thin films with high enzyme loadings: glucose concentration 63 mmol dm^{-3}. The film was grown galvanostatically from a solution containing 2.08 mg ml^{-1} of enzyme; total charged passed 25 mC.

$lk'_D/D_{B'} \gg 1$ we can obtain a good estimate of j_B from the intercept of a plot of j_{obs} against $W^{-1/2}$. Figure 7 shows a typical plot of this type.

Taking the values of j_B we can compare these to the theory by constructing log/log plots of the response as a function of enzyme concentration and the equivalent dimensionless form from eqn. (3). This is shown in Fig. 8: the inset shows the experimental responses as a function of enzyme concentration with the solid line drawn according to eqn. (3). There is excellent agreement between the theory and experiment for all but the very lowest enzyme concentrations.

Deviations from the theory are observed at low enzyme loading or with very thin films, see above. The deviations observed in the variation with film thickness for the thin films arise because we have taken the charge passed as a measure of film thickness. This is not a good assumption for very thin films since we have made no allowance for the non-Faradaic component of the transient current which will be a significant portion of the total charge in this case.

For the case of the variation of response with enzyme loading the non-Faradaic contribution does not distort the results because the same charge is passed to grow each film. The increased response observed in this case at low enzyme loadings arises from adsorption of enzyme at the platinum surface. When the clean platinum electrode is placed in the *N*-methylpyrrole solution containing glucose oxidase the enzyme adsorbs on the electrode surface over a period of approximately 5 min. This

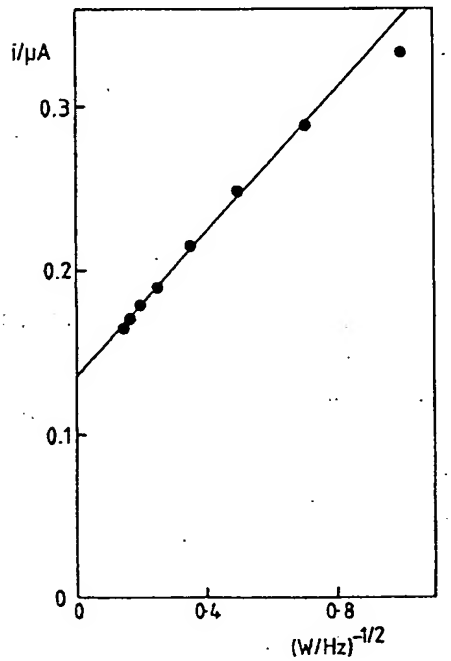

Fig. 7. Plot of the data in Fig. 6 as a function of $W^{-1/2}$ (eqn. 7): the intercept gives a good estimate of j_B.

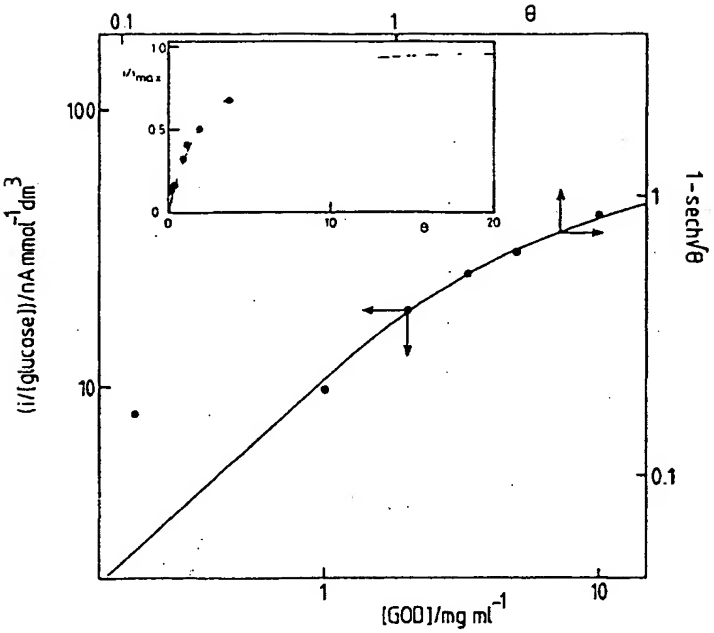

Fig. 8 Comparison of the experimental variation of the response of the electrode to added glucose as a function of the enzyme concentration in the polymerisation solution with the theoretical expression. The points are the experimental results corrected for rotation speed variation as described in the text; the solid curve is calculated from eqn. (3). The values of the kinetic parameters derived from the fit are given in Table 2 The inset shows the fit of experiment to theory. All films were grown galvanostatically total charged passed in the film growth was 40 mC.

leads to an especially large concentration of enzyme at the surface which is still active. In those cases where the glucose penetrates throughout the film this leads to the anomalously large response observed. The adsorption of glucose oxidase at electrode surfaces has also been observed by Bourdillon et al. [10]. Evidence for this is provided by the fact that the responses to glucose for such films depend upon the time between placing the electrode in the solution at open circuit and starting to grow the film. If the electrode is connected to the galvanostat and immersed in the solution so that the electrochemical polymerisation starts immediately, the results are in good agreement with the theory and the deviations are no longer observed.

These results show that the oxidation of hydrogen peroxide is occurring at the platinum surface and not on the conducting polymer backbone. From the theoretical analysis we can determine the kinetic characteristics of the immobilised enzyme and the transport of glucose in the film. These results are summarised in Table 2. We can compare the values derived from the two different experiments. From the results in Table 2 we obtain values of $D_S K/\text{cm}^2\ \text{s}^{-1}$ of 3.4×10^{-11} and 0.4×10^{-11} from the results in Figs. 5 and 8 respectively. From the value of X_K given in Table 2 and using eqn. (4) we find that $(k_{cat}\alpha/D_S K_M)/\text{cm mg}^{-1} = 2.1 \times 10^9$ which compares favourably with the value of 5.6×10^9 derived from Fig. 8.

TABLE 2

Kinetic parameters derived from the fit of experiment to theory

$X_K/D_S K = 6.2\times10^5\ \mathrm{cm^{-1}\ s}$ [a]
$X_K = 2.2\times10^{-5}\ \mathrm{cm}$ [b]
$1/D_S K = 2.5\times10^{11}\ \mathrm{cm^{-2}\ s}$ [c]
$k_{cat}\alpha/D_S K_M = 5.6\times10^9\ \mathrm{cm\ mg^{-1}}$ [d]

[a] Calculated from the shift between experiment and theory in the y-axis in Fig. 5 using $A = 0.48\ \mathrm{cm^2}$; $n = 2$.

[b] Calculated from the shift between experiment and theory in the x-axis in Fig. 5. The thickness was estimated using the literature result [11] that 8 mC $\mathrm{cm^{-2}}$ is equivalent to a thickness of 2×10^{-6} cm.

[c] Calculated from the shift between experiment and theory in the y-axis in Fig. 8. The thickness was estimated as described above.

[d] Calculated from the shift between experiment and theory in the x-axis in Fig. 8. The parameter α describes the relation between the concentration of enzyme in the polymerisation solution, [ez], in mg $\mathrm{ml^{-1}}$ and the final concentration of enzyme in the film, e_Σ; where $e_\Sigma = \alpha$[ez].

The stability of the electrode was investigated by measuring calibration curves for an electrode over a period of 5 days. Between each measurement the electrode was stored in phosphate buffer at room temperature. Over this period the response decreased by 30% of its initial value. At the end of 5 days the electrode failed when the film became detached from the platinum surface.

CONCLUSIONS

The results for glucose oxidase immobilised in electrochemically grown films of *N*-methylpyrrole are in excellent agreement with theory. The oxidation of the hydrogen peroxide formed by the enzymatic reaction occurs at the platinum electrode surface and not on the polymer backbone. This is a simple and advantageous method for the preparation of enzyme electrodes.

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